

**Transplantable cell**

5 The invention relates to a human or animal non-totipotent cell which contains at least one nucleic acid which codes for at least one immune modulator under the control of a gene switch molecule which can be regulated by adding an active substance. The invention also relates to the production and use of said cell for transplantation, for inhibiting transplant rejection and also for the prophylaxis and/or therapy of diseases resulting from a transplant and/or of autoimmune diseases.

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A multiplicity of human diseases are based on the dying or malfunction of specific cells, tissues or organs and can often be treated only insufficiently with medicaments. The conventional therapy previously consisted of replacing the damaged tissue or organ by transplanting healthy cells, tissues or organs such as, 15 for example, heart, lung, kidney, pancreas, or cells or tissues from said organs, which had been obtained from healthy human donors. Owing to the current shortage of organ donors, however, the demand for donor tissue can be met only insufficiently. Said shortage could be eliminated by transplanting tissues or cells from specially bred nonhuman donor mammals. Alternatively, replacement cells 20 may also be obtained from cell lines. These cells may likewise be of human or nonhuman origin.

However, each transplantation of nonhuman cells, tissues or organs into a human organism (xenotransplantation) or of human donor cells, donor tissue or donor 25 organs of a genetically nonidentical human being into a human recipient (allo-transplantation) has the problem of immune mediated transplant rejection. Said rejection is based on the histocompatibility antigens present on the donor cells being recognized as foreign proteins, thereby causing an immune response directed against the transplant. The immune response against allogeneic and xenogeneic 30 cells, tissues and organs is based on numerous complex interactions between different cells (e.g. B cells, T cells, antigen-presenting cells) of the immune system and may ultimately result in the rejection of the transferred cells, tissue or organ. In order to suppress said immune reactions, the recipient must therefore be treated 35 with medicaments, known as "immune modulators", which suppress the immune system or bring about a tolerance of said recipient toward the transplant and thus prevent such a rejection.

Examples of immune modulators used are steroids (prednisolone and derivatives), calcineurine inhibitors (cyclosporine A, tacrolimus), rapamycin (sirolimus), mycophenolate mofetil (MMF), azathioprine (Imuran), lymphocyte antisera (ALG - anti-leukocyte globulin, ATG - anti-thymocyte globulin) or monoclonal 5 antibodies (anti CD25: Zenapax, Simulect).

While antibody therapies are administered as supportive therapies during the first weeks and months after transplantation (induction therapy), calcineurine inhibitors, steroids and, frequently, MMF or Imuran are usually administered from the time of 10 transplantation over the entire period in which the patient carries the transplant, i.e. sometimes all his life. Independently of the type, mode of action and combination of the currently used immune modulatory substances, however, the medicaments are usually administered intravenously or orally and thus distributed over the entire 15 body of the patient. Thus they reach not only their site of action, i.e. the transplanted organ or tissue, or the site at which the transplanted cells have taken, but also all other tissues and organs of the organism. This results in a general suppression of the immune defenses, which is not limited to the transplant. In the non-target tissues and organs, they impair the function of the immune system and thus prevent the physiological functioning of many organs, and this may result in 20 numerous side effects.

The most important side effects of conventional immune modulatory therapy are in this connection hypertension, damage to the kidneys and the liver, the increased occurrence of opportunistic infections and an increased rate of various malignant 25 degenerations such as, for example, cancer and lymphoproliferative disorders. Thus, for example, cytomegalovirus infection which normally proceeds with only slight symptoms may result in the development of hepatitis, pneumonias and meningitides in immunosuppressed patients and is thus a major cause of the increased mortality rate of transplant receivers (Transplantation Clinical 30 Management, Vol. 5, 2000 Medscape, Inc., Web MD Health Network, NY, USA). Other studies confirm that immunosuppressed allograft recipients, after conventional treatment, run a three times to four times higher risk of cancer, which, for particular types of cancer, may even increase by a factor of 20-500 (Penn I., Clin. Transpl. 147-158, 1998). In addition, immune modulators, in 35 particular immunosuppressives, may in general have toxic properties, independently of their action on the immune system. Thus, calcineurine inhibitors frequently cause kidney failure, hypertension, hyperlipidemia and the development

of diabetes mellitus. The regular treatment with medicaments furthermore greatly impairs the quality of life and is therefore frequently not carried out by the patients to the extent which is required medically.

- 5 A further disadvantage is the fact that a relatively high dosage of said medicaments needs to be administered in order for them to still attain the therapeutically active concentration at the site of transplantation, after distribution over the entire circulation. This further promotes or increases the occurrence of said side effects.
- 10 In order to counteract these facts, numerous novel immunotherapeutic and accompanying diagnostic approaches have been developed in recent years.

A clinically tested approach of reducing the side effects of immune modulation, for example, consists of combining newly developed immunoreactive medicaments at 15 low concentrations and replacing standard therapeutics (e.g. steroids, cyclosporine A) therewith. This has been carried out successfully, for example, in the transplantation of allogeneic islet cells for the treatment of diabetes mellitus, where the diabetogenic action of glucocorticoids was circumvented using a combination of daclizumab, sirolimus and low dose tacrolimus (Shapiro J., The 20 New England Journal of Medicine, Vol. 343, N. 4, 230-238, 2000). However, even with this treatment, it was not possible to avoid the side effects of the individual immune modulators of said combination, such as, for example, decrease in the leukocyte level, the occurrence of mouth ulcers and indigestion.

25 Another possible therapeutic approach is to achieve an immunological tolerance to the foreign tissue in the patient by administering novel immune modulatory substances. Tolerance, in this connection, is characterized as the absence of an immune response to or rejection of the transplant without continual immunosuppression. Immune modulatory substances of this kind are administered 30 according to the usual methods known to the skilled worker (see, for example, WO 00/12138; WO 97/41232; WO 96/26274; WO 01/87330; Ferrari-Lacraz et al., The Journal of Immunology, 2001, Vol. 167 pp. 3478-3485; Kim et al., The Journal of Immunology, 1998, 160: 5742-5748; Penn, Transplant Proc, 1991, 23:1101; Beveridge et al., Lancet, 1984, 1:788).

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One disadvantage of the conventional therapeutic approach is the fact that tolerance-inducing immunomodulators are normally also administered

systemically (e.g. intravenously). Another disadvantage is the fact that the immune modulators must either be isolated as therapeutic products from natural sources or be produced biotechnologically as recombinant molecules, in order to administer them to the patient externally thereafter. However, aspects due to production may 5 result in an immune modulator not being able to be isolated or produced in native form or as recombinant molecule in a sufficient quantity or with sufficient activity. In addition, ex vivo production of the immune modulator may require the addition of substances which may pose a further health risk for the patient (e.g. substances which have been isolated from animal organisms and may therefore potentially 10 transfer zoonoses). As a consequence of this, it may not be possible for all patients to be treated with the immune modulator in an adequate manner or the treatment may be associated with additional health risks.

15 It was the object within the scope of the present invention to develop an immunotherapy which avoids or reduces the above-described disadvantages, which, in particular, displays its immune modulatory, in particular immunosuppressive, action in the organism exactly where the immune response has been or can be activated, i.e. at the location of the transplanted cells or organs, and which, at the same time, makes possible an immune modulatory action whose timing and 20 amount can be regulated.

According to the invention, it was possible to control expression of the immune modulator in cells with the aid of a regulatable gene expression system.

25 It is possible, with the aid of said regulatable gene expression system, to produce an immune modulator locally (e.g. in cell transplants) and in doses. Such a regulatable production of immune modulators has not been demonstrated previously. Thus the finding of obtaining in transient cell culture experiments regulatable production of the immune modulator Mutil-15/mFc was all the more 30 surprising (see example 1 and Kim et al., I. Immunology 1998, 160, 5742-5748).

Said regulatable expression system makes it possible that immune modulators need no longer be administered continually and systemically, or only at low concentrations.

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Another considerable advantage of the invention is the fact that, even at the location of the transplant, continual suppression of the immune response need not

take place, if this is not required from a medical point of view, but that said suppression is activated only when needed. This reduces side effects and also strain on the body, in particular on the transplant region. It also means physical and psychological relief for the patient, since he need not continually rely on the

5 administration of immune modulators, in particular immunosuppressives. Immunosuppressives in this context mean substances which completely or partially inhibit an immune response, caused by the transplant, in particular cell(s), tissue and/or organ(s), in the organism.

10 In addition, the present invention provides the advantage that the immune modulator need no longer be isolated from a native source or recombinantly produced and purified. The immune modulator is produced in a therapeutically active amount and form in the organism of the recipient and/or at the site of action in vivo and thus retains its full native activity.

15 One aspect of the present invention therefore relates to a transplantable human or animal non-totipotent cell, comprising at least one nucleic acid coding for at least one immune modulator under the control of a gene expression system which can be regulated by adding an active substance.

20 A nucleic acid means in accordance with the present invention an RNA or DNA, in particular genomic DNA, recombinantly produced DNA, cDNA or synthetic DNA synthesized, for example, at the level of phosphoamidation. Likewise comprised are combinations and/or modifications of nucleotides of said nucleic acids. Said term furthermore comprises single- and double-stranded nucleic acids.

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30 Also comprised are nucleic acids which include functionally linked components, for example one or more genes or active portions thereof, coding for one or more immune modulators and also at least one regulatable gene expression system whose state of activation is regulated by adding an active substance, and also regulatable elements such as, for example, promoters and regulatory nucleotide sequences and also a polyadenylation signal, for example an SV40 polyadenylation signal. Said components are functionally linked if they are connected in such a way that the sequence(s) of the genes or of the gene contained is(are) transcribed under the influence of transcriptional regulation.

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The term immune modulator of the present invention comprises essentially any

type of molecule having immune modulatory, in particular immunosuppressing, action, for example proteins, fusion proteins and soluble ligands, a fusion protein meaning an expression product of a fused gene produced by the linkage of two or more genes or gene fragments.

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An immune modulating action is present if the immune response of an organism, of a cell and/or of a tissue is essentially inhibited, for example by way of an altered or suppressed receptor binding. An immune modulating action is likewise present if an immunological tolerance toward a transplanted cell, tissue or organ is brought about.

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The action of said immune modulator comprises, for example, one or more of the following activities:

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inhibition of an antigen recognition mediated by T cells,

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inhibition of a signal mediated via a receptor on a T cell,

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activation of a signal mediated via a receptor on a T cell,

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inhibition of the growth of T cells,

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inhibition of molecules supporting the survival of T cells,

inhibition of effector molecules of T cells (such as TNF-alpha, IFN-gamma),

inhibition of the adhesion of T cells,

inhibition of a T cell-costimulatory interaction (activation of a lymphocyte takes place via two signals: firstly, a stimulation via the antigen receptor takes place, secondly, another signal for clonal expansion and differentiation of a non-imprinted lymphocyte occurs; this costimulatory interaction may be inhibited by an immune modulator),

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inhibition of the activation, proliferation, survival, antigen presentation, signaling, and/or the effector functions of further cells involved in an immune response, such as, for example, common and special antigen-

presenting cells, in particular, for example, dendritic cells and monocyte/macrophage B cells, neutrophilic granulocytes and NK cells, inhibition of the cellular interaction of different cells, either via surface receptors or via secreted molecules such as, for example, cytokines, chemokines or growth factors, which are involved in an immune response, such as, for example, common and special antigen-presenting cells, in particular, for example, dendritic cells and monocytes/macrophages, T cells, B cells, neutrophilic granulocytes and NK cells,

inhibition of the migration of cells involved in an immune response, such as, for example, special antigen-presenting cells, in particular, for example, dendritic cells and monocytes/macrophages, T cells, B cells, neutrophilic granulocytes and NK cells,

inhibition of components of the complement system,

inhibition of phagocytotic activities in connection with the presentation of foreign or autoimmune antigens, or by binding of antibodies to antigens, and/or

inhibition of inflammatory reactions.

Examples of suitable immune modulators are antibodies. Particular preference is given to an antibody to IL-15, IL-1, IL-2, IL-6, IL-7, IL-12, IL-17, IL-18, IL-21, interferon gamma, TNF-alpha, CD2, CD3, CD4, CD8, CD28, CD40, CD80, CD86 or CD154 or to their receptors.

Preference is likewise given to the immune modulators FasL, PD-L1 or PD-L2.

Further preferred immune modulators are IL-15, IL-10, IL-4, IL-2, interferon gamma or TGF-beta, in particular in the form of a fusion protein. The fusion protein particularly preferably comprises firstly wild type IL-15, wild type IL-10, wild type IL-4, wild type interferon gamma or wild type TGF-beta and, secondly, an Fc fragment. Particular preference is furthermore given to said fusion protein comprising, firstly, mutated IL-15, preferably those in which "Q" in positions 101 and 108 has been replaced with "D", or mutated IL-2 and, secondly, an Fc fragment (see, for example, WO 97/41232; Kim et al., J. Immunol. (1998),

160(12): 5742-5748; WO 01/87330) which is fused, for example, to the C terminus of the mutated IL-15 molecule via the hinge region.

5 Further preferred immune modulators are fusion proteins of, firstly, TNF-alpha receptor (type 1 or 2), ICOS, CTLA-4, PSGL-1, ICAM-1 or VCAM-1 and, secondly, an Fc fragment such as, for example, those disclosed in EP 417,563 in the case of the TNF receptor fusion proteins.

10 Further preferred immunomodulators are secreted variants of cytokine receptors or growth factor receptors, such as, for example, IL-15Ralpha, IL-6, IL-7, IL-12, IL-17, IL-18 receptors, for example as variants without transmembrane domain and cytoplasmic tail, preferably as a fusion protein containing an Fc fragment.

15 The fusion protein is preferably a chimeric fusion protein. Examples of suitable fusion proteins are IL-15 derivatives, comprising IL-15 or mutated IL-15 and a heterologous Fc fragment.

20 An Fc(fragment, crystallizable) fragment means the fragment of an antibody, which does not bind any antigens, for example a fragment which comprises all constant domains or all constant domains with the exception of the first constant (partial or complete) domain, such as, for example, one which comprises the hinge region, the second (CH2) and third (CH3) constant domains of the heavy chain. The Fc fragment may derive from a natural source, be produced recombinantly and/or synthesized. Corresponding methods are known to the skilled worker. In 25 this connection, the Fc fragment may also have one or more mutations compared to the natural sequence, for example those which include a suitable cleavage site for constructing a fusion protein (see Kim et al. *supra*).

30 In a further embodiment of the invention, the Fc fragment is one of an immunoglobulin (Ig)G, in particular a human IgG1, IgG2, IgG3, IgG4 and/or an analogous mammalian IgG and/or an IgGM, in particular a human IgM or an analogous mammalian IgM and/or a murine IgG2a.

35 The immune modulators may have wild type sequences or else mutated sequences. Preferably, the immune modulators are already present in a functionally active form, for example in a functionally active soluble form or a functionally active viral form. A soluble form means a molecule which is not bound to a cell

membrane, such as, for example, a soluble receptor molecule. A viral form means a protein isoform which is encoded endogenously by a viral genome, such as viral IL-10, for example.

5 A mutated sequence according to the present invention means a nucleotide or amino acid sequence which contains deviations from the wild type sequence, for example due to deletion, addition, insertion or substitution of one or more nucleotides or amino acids, but without completely losing the immune modulatory action.

10 Mutated immune modulators in accordance with the present invention are therefore molecules which have a sequence homology to the wild type sequence of preferably at least approximately 80%, preferably at least approximately 90%, particularly preferably at least approximately 95%, most preferably at least 15 approximately 99%.

Sequence homology in accordance with the present invention means the degree of similarity (% positives) of two sequences, which, in the case of polynucleotides, is determined, for example, with the aid of BLASTN 2.0.14, with the filter set to 20 "off" and BLOSUM = 62 (Altschul et al., 1997, Nucl. Acids Res., 25: 3389-3402). The sequence homology may be checked using common sequence homology programs, for example on the Internet under <http://www.hgsc.bcm.tmc.edu/SearchLauncher/>.

25 The term regulatable gene expression system in accordance with the present invention means the combination of a sequence coding for a gene switch molecule and a gene switch binding sequence, the binding of said gene switch molecule to said gene switch binding sequence being regulated by adding an active substance, thereby controlling expression of a target gene, in this case a target gene coding for 30 an immune modulator (see also Burcin et al., 1998, Frontiers in Bioscience 3: cl-7).

In general, it is possible for a gene switch molecule to activate or inhibit transcription of the target gene by binding to a suitable gene switch binding sequence. The activation may be based on the gene switch molecule providing, for 35 example, contact sites for RNA polymerase and/or transcription factors involved. Inhibition may be caused by the gene switch molecule blocking, by way of its binding to the DNA, the DNA binding sites required for the transcription complex

and thereby rendering said binding sites inaccessible to, for example, RNA polymerase and/or to transcription factors involved.

5 The addition of an active substance may influence transcription of the target gene positively (activation) or negatively (inhibition). For example, the target gene is not expressed in the absence of the active substance. After addition of the active substance, the latter binds to the gene switch molecule and thereby causes activation and binding of said gene switch molecule to the gene switch binding sequence, whereby subsequently transcription of the target gene is initiated.

10 Another example is the gene switch molecule binding to the DNA in the absence of the active substance and activating transcription. After addition and binding of the active substance, the gene switch molecule is inactivated and transcription of the target gene is stopped.

15 The term gene switch molecule means a molecule, preferably a protein, in particular a fusion protein, containing a binding site for an active substance and a transcription activation domain, which molecule alters its state of activation after binding of the active substance.

20 The term gene switch binding sequence in accordance with the present invention means preferably a nucleic acid sequence located 5' upstream of the translation start (+1) of the gene or an active portion thereof, coding for an immune modulator, which nucleic acid sequence controls transcription of the target gene, in particular with respect to the rate of transcription and/or tissue specificity, or

25 controls translation. A regulatory nucleic acid sequence having promoter activity, preferably likewise having enhancer activity, is bound to said gene switch binding sequence indirectly or directly.

30 The function of the regulatable gene expression system of the invention may be described as follows, for example:

If no gene switch molecule is bound to the gene switch binding sequence, the coupled target gene is not expressed and, in this case, no immune modulator is produced.

35 When an active substance is added, the latter binds to the dimerization domain of the gene switch molecule, for example. This binding produces a conformational

change in the dimerization domain, which causes dimerization of two gene switch molecules and subsequent binding to the gene switch binding sequence. This binding puts the activation domain of the gene switch molecule in proximity of the minimal TATA promoter and thus initiates transcription of the coupled target gene  
5 coding for an immune modulator. After the active substance has been added, binding of the gene switch molecule to the gene switch binding sequence is dissolved, thus stopping expression of the target gene.

Accordingly, the gene switch molecule of the present invention can be regulated,  
10 for example inhibited or activated, preferably activated, by adding an active substance and then binds to the gene switch binding site.

Preferred active substance means a pharmacologically compatible substance which causes directly or indirectly regulated expression of a gene or a plurality of genes  
15 via its/their gene switch(es), for example mifepristone, tetracycline, doxycycline or rapamycin.

The regulated gene expression system of the present invention is in particular a progesterone gene expression system which comprises a gene switch representing  
20 an artificially composed transcription factor, consisting of a GAL4-DNA binding domain (Gal40-DBD), a dimerization domain derived from a mutated progesterone receptor having a truncated ligand binding site (hPR-LBD), and an activation domain of the p65 subunit of the human NF- $\kappa$ B protein (p65-AD). The gene switch binding sequence is a nucleotide sequence consisting of, for example, a 17  
25 nucleotide GAL4 binding sequence followed by the minimal TATA promoter to which the corresponding target gene is coupled. A regulatable gene expression system of this kind having the mentioned gene switch components Gal4-DBD/hPR-LBD/p65-AD is described, for example, in Wang et al., 1994, PNAS 91: 8180-8184 or Wang et al., 1997, Gene Therapy 4: 432-441. An example of the  
30 active substance suitable in this system is mifepristone, an artificial hormone-like molecule which does not occur in mammals and which binds specifically to the dimerization domain of the gene switch molecule and activates the latter by the dimerization caused thereby.

35 A further regulatable gene expression system of the present invention is a tetracycline gene expression system which represents a system inducible by tetracycline (Tet) or the derivative doxycycline (Dox), in which system the gene

switch molecule consists of a Tet transactivator protein. Said transactivator (tTA) is a fusion protein of a VP16 activation domain and the Tet repressor (TetR) of *Escherichia coli*. In the absence of tetracycline, the tTA has a high affinity for its gene switch binding site, the Tet-responsive element (TRE), and activates expression of the target gene. Addition of the active substance tetracycline inhibits DNA binding and thus target gene activation. This regulatable gene expression system is described, for example, in Gossen 1995, Science 268: 1766-1769; Fruh 1995, Nature 375: 415-418; Chao et al., 1998, Mol. Cell. Biol. 18 (8): 4883-4898; Halappnavar et al., 1999, J. Biol. Chem. 274 (52): 37097-37104 or van der Vlag et al., 2000, J. Biol. Chem. 275 (1): 697-704.

A modification of this regulatory Tet system is the reverse transactivator (rtTA). In this system, wild type TetR has been replaced with a mutated TetR (rTetR), and, as a result, the fusion protein binds the gene switch binding site of the DNA only in the presence of doxycycline and thus induces coupled target genes, as described, for example, in Gossen 1995, Science 268: 1766-1769; Gossen et al., 1992, PNAS 89: 5547-5551; Linstedt et al., 1997, Mol. Biol. Cell 8: 1073-1087; Mehlen et al., 1998, Nature 395: 801-804 or Joosse et al., 2000, Hum. Mol. Genet. 9: 3075-3082.

20 A further regulatable gene expression system of the present invention is a rapamycin gene expression system. This comprises inducible dimerization of the proteins FKBP12 and FRAP, which is mediated by the active substance rapamycin. Dimerization of these two proteins causes DNA binding of the activation domain bound to FRAP and thus switching-on of a coupled target gene. A regulatable gene expression system of this kind is described, for example, in Rivera et al., 1996, Nature Med 2: 1028-1032.

Said active substance may be administered by methods familiar to the skilled worker, for example intravenously, intraperitoneally, intramuscularly, 30 subcutaneously, intracranially, intraorbitally, intracapsularly, intraspinally, transmuscularly, topically, orally or via the mucous membrane, for example the nose or oral cavity. Further examples of methods of administration are systemic or local injection, perfusion or catheter-based administration. Examples of a suitable oral dosage form are tablets or capsules. Administration via the lung is carried out, 35 for example, with the aid of sprays and via the skin in the form of dispositories implanted under the skin. Transdermal therapeutic systems (TTS) are disclosed, for example, in EP 0 944 398-A1, EP 0 916 336-A1, EP 0 889 723-A1 or EP

0 852 493-A1.

The cells are preferably transplantable. Transplantable in accordance with the present invention means that the living cell can be transferred to a different site of 5 the same organism or into a different (recipient) organism, said cell being preferably a nontumorigenic cell or, if the cell is derived from a tumor cell, said cell being treated appropriately prior to transplantation (e.g. by mitotic inactivation) in order to inhibit its proliferation (see, for example, WO 00/64459 and US 5,175,103; Pleasure et al. (1992), The Journal of Neuroscience, 12(5): 10 1802-1815).

The transplantable human or animal non-totipotent cell of the invention is in particular a mammalian cell, including a human cell, and is derived, for example, from humans, mice, rats, guinea pigs, rabbits, cattle, sheep, goats, horses, pigs, 15 dogs, cats or monkeys, preferably from humans.

Examples of cells of the invention are epithelial cells, endothelial cells, liver cells, heart cells, skin cells, muscle cells, nerve cells, bone marrow cells, bone cells, cartilage cells, blood cells, connective tissue cells and cells of the pancreas, the 20 kidney, the eye or the lung.

A non-totipotent cell means a cell which is incapable of developing independently into its complete organism.

25 In a further embodiment, the cell is a stem cell, a precursor cell and/or an immortalized cell. It is preferably a pluripotent or multipotent embryonic, fetal, neonatal or adult stem cell. Particularly preferred stem cells which are derived from adult tissue but are not limited thereto comprise neuronal stem cells, stem cells of the bone marrow, mesenchymal stem cells, hematopoietic stem cells, 30 epithelial stem cells and also stem cells of the digestive tract, the skin, the adipose tissue, the intestine, the placenta and the pancreatic duct.

A cell of the present invention also comprises a cell which contains the above-described of the invention and which is introduced into a tissue or an organ of a 35 human or animal organism, before and/or after said tissue or organ is transplanted into the same or a different human or animal organism.

A preferred embodiment relates to a cell of the invention in the form of a cell line.

A cell line of the invention may be prepared, for example, by transforming or infecting a cell line with the above-described nucleic acid of the invention with the aid of methods familiar to the skilled worker, for example transfection, transformation or infection.

In a further embodiment of the invention, the nucleic acid additionally encodes a selection cassette, in particular a suitable transfection marker gene and/or differentiation marker gene.

A selection cassette in accordance with the present invention is a nucleic acid sequence coding for at least one gene which causes specific selection of particular cells, for example transfected or differentiated cells.

It is possible to use for a selection of this kind, for example, differentiation marker genes, transfection marker genes and reporter genes. Genes used as such are primarily genes which mediate a resistance to particular toxic substances, for example antibiotics. The most frequently used antibiotics in this connection are neomycin, hygromycin (hph), zeocin (Sh ble) and puromycin (pacA). Other examples of genes of this kind, in particular for selecting stem cells, are, for example, genes which regulate expression of fluorescent markers, for example GFP, with the aid of which the cells to be selected can be purified via fluorescence-assisted cell sorting (FACS). Other examples of selection markers are surface molecules, for example growth factor receptors, with the aid of which cells may be enriched via magnetic immunobeads (Bonini C., Science Vol 276, 1719-1724, 1997). Other examples are genes coding for an enzyme activity (e.g. thymidine kinase), which convert a precursor of a toxic substance, a "prodrug" (e.g. ganciclovir) to a toxic substance. In this case, negative selection may take place, i.e. only the cells which do not express the promoter upstream of the gene survive.

Further possible genes of the selection cassette of the invention are lacZ (coding for  $\beta$ -lactamase),  $\beta$ -lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), dihydrofolate reductase (DHFR) and xanthine guanine phosphoribosyl transferase (XGPRT). The skilled worker is familiar with reagents which may, where appropriate, be used in order to ensure or increase the

function of said genes, for example additional nucleic acid sequences.

5 In a preferred embodiment, the nucleic acid additionally encodes a molecule inhibiting NK cells and/or killer cells, preferably a human MHC class I molecule, a chimeric MHC class I molecule or a viral MHC class I homolog.

10 Killer cells are known by the skilled worker as a heterogeneous population of mononuclear cells with spontaneous or acquired cytotoxic potential. NK cells (natural killer cells) mean killer cells which are naturally present, i.e. which are not the result of an immune response and are thus not induced antigen-specifically.

15 The present invention further relates to a nucleic acid which codes for at least one immune modulator and at least one gene expression system which can be regulated by adding an active substance, as already described in more detail above.

15 The nucleic acid preferably likewise codes for at least one element controlling gene expression. These elements mean, for example, promoters or regulative nucleic acid sequences. These as well as expression vectors (illustrated in more detail below) may create suitable conditions for expressing a nucleic acid. 20 Expression vectors generally comprise promoters suitable for the particular cell or for the gene to be transcribed in each case.

25 Examples of regulatable elements enabling constitutive expression in eukaryotes are promoters recognized by RNA polymerase III. Promoters of this kind for constitutive expression in all cell and tissue types are, for example, the pGK (phosphoglycerate kinase) promoter, the CMV (cytomegalovirus) promoter, the TK (thymidine kinase) promoter, the EF1 $\alpha$  (elongation factor 1-alpha) promoter, the SV40 (simian virus) promoter, the RSV (Rous sarcoma virus) promoter and the pUB (ubiquitin) promoter.

30 Examples of regulatable elements enabling cell- or tissue-specific expression in eukaryotes are promoters or activator sequences of promoters or enhancers of those genes coding for proteins which are expressed only in particular cell types. Examples of such promoters are the insulin promoter for pancreatic beta cells, the 35 Sox-2 promoter for nerve cells, the myosin heavy chain promoter for muscle cells, the VE-cadherin promoter for endothelial cells and the keratin promoter for epithelial cells.

Further examples of regulatable elements enabling regulatable expression in eukaryotes are the tetracycline operator combined with a corresponding repressor (Gossen M. et al. (1994) Curr. Opin. Biotechnol. 5, 516-20).

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Expression may likewise be controlled via regulative nucleotide sequences which influence expression with respect to quantity and/or as a function of time. They include, for example, enhancer sequences, leader sequences, polyadenylation sequences, IRES sequences, introns, insulator sequences and repressor sequences.

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The nucleic acid of the invention may be located on one or more nucleic acid molecules. In the case of a plurality of nucleic acid molecules, however, these must functionally cooperate according to the invention. It is possible for example, in accordance with the present invention, that (i) the sequence for the gene switch molecule, (ii) the sequence for the immune modulator under regulation of the gene switch binding site and (iii) the sequences for the selection markers are located on three different nucleic acid molecules. Due to transcription of the gene switch molecule in the nucleus, the gene switch protein produced in the cytoplasm after translation may in turn bind in the nucleus to the gene switch binding site of the immune modulator sequence and regulate expression thereof.

The present invention therefore furthermore also relates to a vector which comprises at least one nucleic acid of the invention.

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Possible vectors in accordance with the present invention are plasmids, shuttle vectors, phagemids, cosmids, adenoviral vectors, retroviral vectors, expression vectors and vectors active in gene therapy.

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Expression vectors in accordance with the present invention comprise at least one nucleic acid of the invention, at least one translation initiation signal, one translation termination signal and/or one polyadenylation signal for expression in eukaryotes.

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Expression vectors of this kind, in particular for expression in mammalian cells, are inter alia commercially available, for example pIRES (Clontech, Heidelberg, DE), pCI-neo vector (Promega, Mannheim, DE), pCMV-Script (Stratagene, La Jolla, USA) and pcDNA3 vector (Invitrogen, Karlsruhe, DE) or may be assembled

individually from individual elements.

Examples of vectors according to the invention, which are active in gene therapy, are plasmid vectors, viral vectors, for example adenoviral vectors, retroviral 5 vectors or vectors based on replicons of RNA viruses (see, for example, Lindemann et al., 1997, Mol. Med. 3: 466-76; Springer et al., 1998, Mol. Cell. 2: 549-58; Khromykh, 2000, Curr. Opin. Mol. Ther.; 2: 555-69).

10 Vectors active in gene therapy can also be obtained by complexing the nucleic acid fragments of the invention with liposomes. Lipofection involves preparation of small unilamellar vesicles from cationic lipids by sonicating the liposome suspension. The DNA is bound ionically to the liposome surface, and in a ratio so as for a net positive charge to be retained and 100% of the plasmid DNA to be complexed by the liposomes. In addition to the lipid mixtures DOTMA 15 (1,2-dioleyloxypropyl-3-trimethylammonium bromide) and DPOE (dioleoyl-phosphatidylethanolamine), numerous new lipid formulations have been synthesized in the mean time and tested for their transfection efficiency in various cell lines (Behr et al., 1989, Proc. Natl. Acad. Sci. USA 86: 6982-6986; Gao and Huang, 1991, Biochem. Biophys. Acta 1189, 195-203; Felgner et al., 1994, J. Biol. 20 Chem. 269, 2550-2561). Examples of said new lipid formulations are DOTAP, (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium ethyl sulfate and DOGS (TRANSFECTAM; dioctadecylamidoglycylspermine). Possible examples of excipients increasing the transport of nucleic acids into the cells are proteins or peptides bound to DNA or synthetic peptide DNA molecules which enable the 25 nucleic acid to be transported into the nucleus (Schwartz et al., 1999, Gene Therapy 6: 282; Branden et al., 1999, Nature Biotech. 17: 784). Excipients also comprise molecules which enable nucleic acids to be released into the cytoplasm of the cell (Planck et al., 1994, J. Biol. Chem. 269, 12918; Kichler et al., 1997, Bioconj. Chem. 8, 213) or, for example, liposomes (Uhlmann and Peimann, 1990, 30 Chem. Rev. 90, 544). The cells of the invention may also be used for expressing a heterologous gene.

Gene therapy vectors may be introduced into cells by transfection (e.g. 35 electroporation, lipofection, calcium phosphate precipitation) or infection.

The present invention further relates to a medicament which comprises at least one cell of the invention and suitable excipients and/or additives.

The medicament of the invention may be used for the prophylaxis and/or therapy of diseases, for example of

- 5 (a) rheumatic disorders, for example rheumatoid arthritis, Sjögren's syndrome, scleroderma, dermatomyositis, polymyositis, Reiter's syndrome or Behcet's disease,
- (b) type I diabetes or LADA,
- (c) autoimmune diseases of the thyroid, for example Graves' disease,
- 10 (d) autoimmune diseases of the central nervous system, for example multiple sclerosis,
- (f) skin diseases, for example psoriasis or neurodermatitis,
- (g) inflammatory bowel diseases, for example ulcerative colitis or Crohn's disease,
- (h) immune disorders,
- 15 (i) vascular diseases and
- (j) diseases resulting from a transplant, for example transplant rejections.

Suitable excipients and additives which serve, for example, to stabilize and/or preserve the medicament, are generally familiar to the skilled worker. They 20 include, for example, physiological saline, Ringer dextrose, Ringer lactate, University of Wisconsin solution/ViaSpan® (Belzer UW), EuroCollins solution, DMSO, ethylene glycol, sucrose, trehalose, Ficoll, perfluorocarbons, demineralized water, stabilizers, antioxidants, complexing agents, antimicrobial compounds, proteinase inhibitors and/or inert gases.

25

The medicament of the invention is administered according to methods suitable for the particular type of cells, tissues or organs, to which they are to be administered. Methods of this kind are familiar to the skilled worker. According thereto, the medicament may be administered, for example,

- 30   · intravenously for liver cells,
- intramuscularly, or else by catheter-based administration, for cardiac muscle cells, and
- subcutaneously, intravenously, intraperitoneally, encapsulated or intra-muscularly for  $\beta$  cells.

35

The medicament may be introduced into the organism either with the aid of an *ex vivo* approach in which the cells are removed from the patient, genetically

modified, for example by DNA transfection, and then reintroduced into said patient or with the aid of an *in vivo* approach in which vectors of the invention, which are active in gene therapy, are introduced into the patient's body in the form of naked DNA or by using viral or nonviral vectors of the invention or cells of the invention.

5

The dosage of medicaments is known to depend on a plurality of factors, for example on the body weight, the general state of health, the size of the body surface, the age of the patient and the interaction with other medicaments. A dosage likewise depends on the type of administration. Therefore, the dosage has 10 to be determined in each individual case by the skilled worker for each patient. The medicament may be administered once or several times per day and over several days; this too can be determined by the skilled worker.

15 The present invention further relates to a human or animal organ-specific tissue and/or a human or animal mammalian organ, which comprises at least one cell of the invention.

20 The terms organ-specific tissue and mammalian organ in accordance with the present invention comprise, for example, the mammalian organs heart, skin, pancreas, kidneys, liver, muscles, nerves, eye, lung, bone marrow, cartilage, bones, vessels, connective tissue and, respectively, tissues of said organs.

25 The present invention also relates to a transgenic nonhuman mammal which comprises at least one cell of the invention.

25

Transgenic animals usually exhibit tissue-specifically increased expression of nucleic acids and are therefore very suitable for analyzing immune reactions, for example. Preference is given to using transgenic mice.

30 Examples of nonhuman mammals of the invention are mice, rats, guinea pigs, rabbits, cattle, sheep, goats, horses, pigs, dogs, cats or monkeys.

35 The present invention further relates to the use of a cell of the invention, of a human or animal organ-specific tissue of the invention and/or of a human or animal mammalian organ of the invention for transplantation into a human or animal mammal. The transplantation of the invention is preferably an auto-, allo- or xenotransplantation.

Transplantation is understood by the skilled worker as transferring or else transplanting living material, for example cells, tissues and organs to a different site of the same organism (autotransplantation) or from one organism (donor) to another organism (recipient). In the case of transplantation to a different organism, a distinction is made between

- synotransplantation in which the donor and the recipient belong to the same species and are genetically fully or substantially identical,
- allotransplantation in which the donor and the recipient belong to the same species but are immunogenetically different, and
- xenotransplantation in which the donor and the recipient do not belong to the same species and are consequently immunogenetically completely different.

15 The present invention further relates to the use of a cell of the invention, of a human or animal organ-specific tissue of the invention and/or of a human or animal mammalian organ of the invention for inhibiting transplant rejection in animal mammals or in humans.

20 Animal mammals in accordance with the present invention mean, for example, mice, rats, guinea pigs, rabbits, cattle, sheep, goats, horses, pigs, dogs, cats or monkeys.

25 Transplant rejection is understood by the skilled worker as being a process by which the transplanted material, for example cells, tissues or an organ, is rejected by the recipient organism. Said rejection is caused by a cellular and a humoral immunity. The cause of said rejection is a difference in protein structure between the transplanted material and the recipient. The protein structure of the transferred tissue is recognized by the immune system of the recipient as being immunogenic, thereby triggering an immune response.

30

35 The present invention further relates to the use of a cell of the invention, of a human or animal organ-specific tissue of the invention and/or of a human or animal mammalian organ of the invention for the prophylaxis and/or therapy of diseases resulting from a transplant and/or autoimmune diseases. Examples of such diseases have already been described hereinbefore in the context of the field of application of the medicament of the invention.

The uses according to the invention for inhibiting transplant rejection and for the prophylaxis and/or therapy of diseases resulting from a transplant and/or of autoimmune diseases may take place, for example, by introducing the cells, tissues and/or organs of the invention into a human or animal mammal. Expression of the immune modulator of the invention is controlled via the regulatable gene expression system of the invention. Said control is carried out by administering or discontinuing administration of an active substance of the invention, thereby activating or deactivating the gene switch molecule. In the case of activation, the gene switch activates transcription of the target gene coding for the immune modulator. Said immune modulator which is expressed as a result essentially inhibits a defensive response of the immune system to the transplanted cell, tissue or organ, especially in the transplant region of the organism of the human or animal mammal.

15 A treatment based on the use of cells may be achieved by selecting cells of the invention from epithelial cells, endothelial cells, liver cells, derivatives of non-totipotent embryonic stem cells or of non-totipotent embryonic germ cells or stem cells derived from adult tissue. Preferred stem cells derived from adult tissue comprise neuronal stem cells, stem cells of the bone marrow, mesenchymal stem cells, hematopoietic stem cells, epithelial stem cells, stem cells of the digestive tract, the skin, the adipose tissue, the intestine, the placenta and the pancreatic duct, which are introduced into a human or animal mammal.

25 The present invention also relates to a process for preparing a cell of the invention, which process comprises the following steps:

- a. introducing at least one nucleic acid of the invention and/or at least one vector of the invention into a transplantable human or animal non-totipotent cell, and
- 30 b. expressing said nucleic acid with addition of at least one suitable active substance for regulating the gene switch molecule.

In order to introduce a nucleic acid, a vector, a differentiation marker gene or a transfection marker gene or a cell according to the present invention into a cell, use is made of the standard methods of transfection, transformation, electroporation or injection which are familiar to the skilled worker.

Suitable conditions causing or enhancing expression of said nucleic acid have already been described hereinbefore. These include, for example, expression vectors, promoters and regulatable nucleic acid sequences, for example enhancers, polyadenylation sequences.

5

The present invention also relates to an *in vitro* process for preparing a human or animal organ-specific tissue of the invention and/or a human or animal mammalian organ of the invention, said process comprising the following steps:

- 10 a. introducing both at least one nucleic acid of the invention and/or at least one vector of the invention and at least one differentiation marker gene into at least one non-totipotent stem cell, a non-totipotent precursor cell and/or a non-totipotent immortalized cell,
- b. differentiating the cell of step a.,
- c. selecting the differentiated cell of step b., and
- 15 d. introducing the selected cell of step c. into a human or animal organ-specific tissue and/or into a human or animal mammalian organ.

In a further embodiment, preference is given to introducing in said *in vitro* process of the invention after, before or simultaneously with step a. at least one suitable 20 transfection marker gene into a non-totipotent stem cell, a non-totipotent precursor cell and/or a non-totipotent immortalized cell and, after step a., preferably selecting the transfected cell of step a.

The differentiation of cells containing the nucleic acid of the invention may be induced, for example, by embroid body formation, preferably by culturing the cells 25 in solutions, by culturing the cells at high density, by cell aggregation, by removing culturing on feeder cells, removing differentiation-inhibiting substances (e.g. LIF or medium conditioned by feeder cells), by adding cytokines, growth factors, hormones, vitamins (e.g. nicotinamide), retinoic acid, sodium butyrate or 30 DMSO to the cultured cells or by adding other substances which are known to initiate differentiation.

Numerous processes for selecting cells are known.

35 Processes for selecting cells from differentiated embryonic stem cells are described, for example, in Klug et al. (J. Clin. Invest. 1996 Jul 1; 98 (1): 216-24) and Soria et al. (Diabetes. 2000 Feb.; 49 (2): 157-62).

In one preferred method of selection, the selection cassette of the invention comprises a marker gene, that is a gene for resistance to an antibiotic. The cells are selected or isolated by concentrating the differentiated cells after adding a suitable 5 antibiotic during or after the differentiation step. Only the differentiated cells which express the marker gene are resistant to the antibiotic. Undifferentiated cells die. Selection of the transfected cells may also be carried out by the same method.

10 An antibiotic of the invention means an antibiotic to which a resistance is generated by the antibiotic-resistance gene(s) used as selection cassette of the invention. After addition of the antibiotic to the cultured stem cells, essentially only those stem cells which contain the reporter gene expression vector survive and differentiate.

15 It is likewise possible to apply a selection process in which the gene or genes of the selection cassette of the invention code for luciferase, green fluorescent protein, red fluorescent protein and/or yellow fluorescent protein. The cells to be selected are isolated by means of fluorescence-activated cell sorting (FACS) or selected by affinity purification.

20 It is furthermore possible to concentrate cells with the aid of surface molecules, for example growth factor receptors, by means of magnetic immunobeads (Bonini C., Science Vol. 276, 1719-1724, 1997).

25 Preferably, a second marker gene may be introduced into the cells, thereby making it possible to carry out a selection of the cells into which the nucleic acid and/or the vector according to step a. of the *in vitro* process of the invention has been introduced successfully. This double selection makes it possible to obtain an approx. 90%, preferably approx. 95-100%, pure population of the desired cells.

30 The present invention further relates to a process for generating a transgenic non-human mammal of the invention, which process comprises the following steps:  
a. introducing both at least one nucleic acid of the invention and/or at least one vector of the invention and at least one suitable transfection marker gene into at least one oocyte, stem cell, precursor cell and/or immortalized cell of a nonhuman mammal,  
35 b. selecting the transfected cell of step a.,

- c. introducing the cell selected according to step b. into at least one nonhuman mammalian blastocyst,
- d. introducing the blastocyst of step c. or the embryo of step d. into a nonhuman mammalian foster mother, and
- 5 e. identifying the transgenic nonhuman mammal developed from said blastocyst.

In a preferred embodiment, the stem cell is a pluripotent or multipotent embryonic, fetal, neonatal or adult stem cell.

10 The present invention further relates to a process for generating a transgenic non-human mammal of the invention, which process comprises the following steps:

- a. introducing both at least one nucleic acid of the invention and/or at least one vector of the invention and at least one suitable transfection marker gene into either of the two pronuclei of a fertilized nonhuman mammalian oocyte,
- 15 b. introducing the mammalian oocyte of step a. into a nonhuman mammalian foster mother, and
- c. identifying the transgenic nonhuman mammal developed from said 20 mammalian oocyte.

Preference is given to the nonhuman mammalian foster mother having been rendered pseudopregnant by mating with a male having undergone vasosection.

25 Processes for introducing blastocysts and/or oocytes into the foster mother are known to the skilled worker. Said introduction may be carried out, for example, by way of injection into the fallopian tube or the uterus (see, for example, Hogan, B., Beddington, R., Constantini, F and Lacy, E., A laboratory Manual (1994), Cold Spring Harbor Laboratory Press, pages 173-181).

30 A transgenic nonhuman mammal may be identified, for example, by extracting genomic DNA from said transgenic nonhuman mammal, for example from the tail of a mouse. In a subsequent PCR (polymerase chain reaction) analysis, primers are used which specifically recognize the transgene for the nucleic acid of the 35 invention. Integration of said transgene into the genome may be detected in this manner.

It is also possible to carry out identification by means of a Southern blot. In this case, genomic DNA is transferred to a membrane and detected by means of DNA probes, for example radiolabeled DNA probes, which are specific for the transgene searched for.

5

Processes for generating a transgenic nonhuman mammal of the invention by regenerating a nonhuman stem cell, oocyte, precursor cell or immortalized cell to give a transgenic nonhuman animal, in particular transgenic mice, are known to the skilled worker, for example from DE 196 25 049 and US 4,736,866; US 5,625,122; 10 US 5,698,765; US 5,583,278 and US 5,750,825 and comprise transgenic animals which may be generated, for example, by direct injection of expression vectors of the invention into embryos or spermatocytes or via transfection of expression vectors into embryonic stem cells (see, for example, Polites and Pinkert: DNA Microinjection and Transgenic Animal Production, pages 15-68 in Pinkert, 1994: 15 Transgenic Animal Technology: A Laboratory Handbook, Academic Press, London, UK; Houdebine 1997, Harwood Academic Publishers, Amsterdam, The Netherlands; Doetschman: Gene Transfer in Embryonic Stem Cells, pages 115-146 in Pinkert, 1994, *supra*; Wood: Retrovirus-Mediated Gene Transfer, pages 147-176 in Pinkert, 1994, *supra*; Monastersky: Gene Transfer Technology: Alternative 20 Techniques and Applications, pages 177-220 in Pinkert, 1994, *supra*).

Numerous processes for preparing transgenic animals, in particular transgenic mice, are likewise known to the skilled worker, *inter alia* from WO 98/36052, WO 01/32855, DE 196 25 049, US 4,736,866, US 5,625,122, US 5,698,765, US 25 5,583,278 and US 5,750,825 and comprise transgenic animals which may be generated, for example, via direct injection of vectors of the invention into embryos or spermatocytes or via transfection of vectors or nucleic acids into embryonic stem cells (see also Polites and Pinkert, in Pinkert, (1994) Transgenic animal technology, A Laboratory Handbook, Academic Press, London, UK, pages 30 15 to 68; Doetschman, in Pinkert, 1994, *supra*, pages 115 to 146).

In further embodiments, the stem cell used in said *in vitro* processes of the invention for preparing a human or animal organ-specific tissue of the invention and/or a human or animal mammalian organ of the invention and in the processes 35 for generating a transgenic nonhuman mammal of the invention is a pluripotent or multipotent embryonic, fetal, neonatal or adult stem cell.

The present invention likewise relates to a transgenic nonhuman mammal which has been generated by the above-described process of the invention and to the descendent(s) of said mammal.

- 5 The present invention further relates to the use of a transgenic nonhuman mammal of the invention for obtaining a human or animal cell, a human or animal organ-specific tissue and/or a human or animal mammalian organ for allo- and/or xeno-transplantation.
- 10 The cell transplantation, for example, may be carried out by means of an implantation process or by means of a catheter injection method through the blood vessel wall.

Obtaining in accordance with the present invention means removing said cell, tissue and/or organ from the organism of a transgenic nonhuman mammal of the invention. Methods of such a removal are familiar.

The present invention further relates to the use of a transgenic nonhuman mammal of the invention, of a cell of the invention, of a human or animal organ-specific tissue of the invention and/or of a human or animal mammalian organ of the invention for finding pharmacologically active principles and/or for identifying toxic substances.

A method of this kind could consist, for example, of seeding cells of the present invention, for example, on a 96-well microtiter plate, followed by adding a pharmacologically active or toxic substance to be studied and then analyzing by means of cell count, whether said substance has caused the death of an increased number of cells.

30 The terms pharmacologically active principle and toxic substance in accordance with the invention mean all those molecules, compounds and/or compositions and substance mixtures which, under suitable conditions, exert a pharmacological or toxic influence on individual cells, individual tissues, individual organs or the entire organism of an animal or human mammal. Possible pharmacologically active principles and toxic substances may be simple chemical (organic or inorganic) molecules or compounds, nucleic acids or analogs of nucleic acids, antisense sequences of nucleic acids, peptides, proteins or complexes and

antibodies. Examples are organic molecules which are derived from substance libraries and which are studied for their pharmacological or toxic activity.

Pharmacologically active principles are, for example, principles which influence:

- 5     · the ability of cells to divide and/or survive,
- the secretion of proteins, for example insulin from pancreatic beta cells, dopamine from nerve cells,
- the contraction of muscle cells and/or
- the migration behavior of cells,
- 10    · the metabolic activity of cells,
- the electrophysiological activity of cells,
- the enzymic activity of cell products,
- cell differentiation,
- the organization of cells into tissues or organs.
- 15    Applied to the entire organism of an animal or human mammal, this means an influence on, for example,
  - the cardiovascular system,
  - the endocrine system,
  - the gastrointestinal system,
- 20    · the nerve system and
  - metabolic activities.

Examples of toxic substances are active principles which

- 25    · stimulate cells to enter apoptosis, following particular signals, for example stress,
- influence the cardiovascular system,
- influence the nerve system and/or
- influence the metabolic activities.

30    The identified pharmacologically active principles and toxic substances may, where appropriate, be combined or used together with suitable additives and/or excipients for preparing a diagnostic agent or a medicament for the prophylaxis and/or therapy of diseases resulting from a transplant and/or of autoimmune diseases, as set out by way of example hereinbefore.

35

The present inventions also relate to:

(i) A human or animal non-totipotent cell, comprising at least one nucleic acid coding for at least one immune modulator under the control of a gene expression system which can be regulated by adding an active substance.

5 (ii) The cell according to (i), characterized in that the cell is a stem cell, a precursor cell and/or an immortalized cell.

(iii) The cell according to (i) or (ii), characterized in that it is a pluripotent or multipotent embryonic, fetal, neonatal or adult stem cell.

10 (iv) The cell according to at least one of (i) to (iii) in the form of a cell line.

(v) The cell according to at least one of (i)-(iv), characterized in that the gene expression system which can be regulated is a progesterone gene expression system, a tetracycline expression system and/or a rapamycin gene expression system.

15 (vi) The cell according to at least one of (i)-(v), characterized in that the immune modulator has at least one of the following functional properties:

20 a. inhibition of an antigen recognition mediated by T cells,

b. inhibition of a signal mediated via a receptor on a T cell,

25 c. activation of a signal mediated via a receptor on a T cell,

d. inhibition of the growth of T cells,

e. inhibition of molecules supporting the survival of T cells,

30 f. inhibition of effector molecules of T cells (such as TNF-alpha, IFN-gamma),

g. inhibition of the adhesion of T cells,

35 h. inhibition of a T cell-costimulatory interaction (activation of a lymphocyte takes place via two signals: firstly, a stimulation via the

antigen receptor takes place, secondly, another signal for clonal expansion and differentiation of a non-imprinted lymphocyte occurs; this costimulatory interaction may be inhibited by an immune modulator),

5

- i. inhibition of the activation, proliferation, survival, antigen presentation, signaling, and/or the effector functions of further cells involved in an immune response, such as, for example, common and special antigen-presenting cells, in particular, for example, dendritic cells and monocyte/macrophage B cells, neutrophilic granulocytes and NK cells, inhibition of the cellular interaction of different cells, either via surface receptors or via secreted molecules such as, for example, cytokines, chemokines or growth factors, which are involved in an immune response, such as, for example, common and special antigen-presenting cells, in particular, for example, dendritic cells and monocytes/macrophages, T cells, B cells, neutrophilic granulocytes and NK cells,
- j. inhibition of the migration of cells involved in an immune response, such as, for example, special antigen-presenting cells, in particular, for example, dendritic cells and monocytes/macrophages, T cells, B cells, neutrophilic granulocytes and NK cells,
- k. inhibition of components of the complement system,
- l. inhibition of phagocytotic activities in connection with the presentation of foreign or autoimmune antigens, or by binding of antibodies to antigens, and/or
- m. inhibition of inflammatory reactions.

(vii) The cell according to at least one of (i) to (vi), characterized in that the immune modulator is an antibody.

35 (viii) The cell according to at least one of (i) to (vi), characterized in that the immune modulator is

- a. a receptor,

- b. a soluble secreted receptor,
- c. a secreted protein or peptide.

5 (ix) The cell according to (viii), in which the immune modulator is a fusion protein of a mutated IL 15 and an Fc fragment, said Fc fragment being fused to the C terminus of the mutated IL 15 molecule, preferably via the hinge region.

10 (x) The cell according to (ix), characterized in that the Fc fragment of the antibody is an Fc fragment of an IgG, in particular of a human IgG1, IgG2, IgG3, IgG4 or an analogous mammalian IgG or an IgM, in particular a human IgM or an analogous mammalian IgM.

15 (xi) The cell according to at least one of (i) to (x), characterized in that the nucleic acid additionally encodes a selection cassette, in particular a suitable transfection marker gene and/or differentiation marker gene.

20 (xii) The cell according to at least one of (i) to (xi), characterized in that the nucleic acid additionally encodes a molecule which inhibits NK cells and/or killer cells.

25 (xiii) The cell according to (i)-(xi), characterized in that the nucleic acid additionally encodes a molecule which inhibits

- a. dendritic cells,
- b. monocytes and/or macrophages,
- c. B cells,
- d. polymorphonuclear cells, for example neutrophilic granulocytes.

30 (xiv) The cell according to (xiii), characterized in that said inhibitory molecule is a human MHC class I molecule, a chimeric MHC class I molecule or a viral MHC class I homolog.

35 (xv) A nucleic acid, coding for at least one immune modulator and at least one gene expression system which can be regulated by adding an active substance.

(xvi) A vector, comprising at least one nucleic acid according to (xv).

(xvii) A medicament, comprising at least one cell according to any of (i) to (xiv) and suitable expedients and/or additives.

5 (xviii) A human or animal organ-specific tissue and/or a human or animal mammalian organ, comprising at least one cell according to any of (i) to (xiv).

10 (xix) A transgenic nonhuman mammal, comprising at least one cell according to any of (i) to (xiv).

15 (xx) The use of a cell according to any of (i) to (xiv) and/or of a human or animal organ-specific tissue and/or of a human or animal mammalian organ according to (xviii) for transplantation into a human or animal mammal.

(xxi) The use according to (xx), characterized in that it is an allo-, auto- or xenotransplantation.

20 (xxii) The use of a cell according to any of (i) to (xiv), of a nucleic acid according to (xv), of a human or animal organ-specific tissue and/or of a human or animal mammalian organ according to (xviii) for preparing a medicament for inhibiting transplant rejection in a human or animal mammal, where appropriate in the presence of at least one immune modulator.

25 (xxiii) The use of a cell according to any of (i) to (xiv), of a nucleic acid according to (xv), of a human or animal organ-specific tissue and/or of a human or animal mammalian organ according to (xviii) for preparing a medicament for the prophylaxis and/or therapy of diseases resulting from a transplant and/or of autoimmune diseases.

30 (xxiv) A process for preparing a cell according to any of (i) to (xiv), which process comprises the following steps:

35     c. introducing at least one nucleic acid according to (xv) and/or at least one vector according to (xvi) into a transplantable human or animal non-totipotent cell, and

   d. expressing said nucleic acid with addition of at least one suitable active substance for regulating the gene switch.

(xxv) An *in vitro* process for preparing a human or animal organ-specific tissue and/or a human or animal mammalian organ according to (xviii), which process comprises the following steps:

5           e. introducing both at least one nucleic acid according to (xv) and/or at least one vector according to (xvi) and as well at least one differentiation marker gene into at least one non-totipotent stem cell, a non-totipotent precursor cell and/or a non-totipotent immortalized cell,

10           f. differentiating the cell of step a.,

              g. selecting the differentiated cell of step b., and

              h. introducing the selected cell of step c. into a human or animal organ-specific tissue and/or into a human or animal mammalian organ.

15           (xxvi) The process according to (xxv), characterized in that, after, before or simultaneously with step a., at least one suitable transfection marker gene is introduced into at least one non-totipotent stem cell, a non-totipotent precursor cell and/or a non-totipotent immortalized cell and, after step a., the transfected cell of step a. is selected preferentially.

20           (xxvii) The process according to either of (xxv) and (xxvi), characterized in that the stem cell is a pluripotent or multipotent embryonic, fetal, neonatal or adult stem cell.

25           (xxviii) A process for generating a transgenic nonhuman mammal according to (xviii), which process comprises the following steps:

30           f. introducing both at least one nucleic acid according to (xv) and/or at least one vector according to (xvi) and at least one suitable transfection marker gene into at least one oocyte, stem cell, precursor cell and/or immortalized cell of a nonhuman mammal,

              g. selecting the transfected cell of step a.,

              h. introducing the cell selected according to step b. into at least one nonhuman mammalian blastocyst,

35           i. introducing the blastocyst of step c. into a nonhuman mammalian foster mother, and

              j. identifying the transgenic nonhuman mammal developed from said

blastocyst.

(xxix) The process according to (xxviii), characterized in that the stem cell is a pluripotent or multipotent embryonic, fetal, neonatal or adult stem cell.

5

(xxx) A process for generating a transgenic nonhuman mammal according to (xix), which process comprises the following steps:

- d. introducing both at least one nucleic acid according to (xv) and/or at least one vector according to (xvi) and at least one suitable transfection marker gene into either of the two pronuclei of a fertilized nonhuman mammalian oocyte,
- e. introducing the mammalian oocyte of step a. into a nonhuman mammalian foster mother, and
- f. identifying the transgenic nonhuman mammal developed from said mammalian oocyte.

15 (xxxi) A transgenic nonhuman mammal, characterized in that it has been generated by the process according to either of (xxviii) and (xxix).

20 (xxxii) A transgenic nonhuman mammal, characterized in that it is a descendent of the mammal according to (xxx).

25 (xxxiii) The use of a transgenic nonhuman mammal according to any of (xix), (xxx) and (xxxi) for obtaining a nonhuman cell, a nonhuman organ-specific tissue and/or a nonhuman mammalian organ for allo- and/or xenotransplantation.

30 (xxxiv) The use of a transgenic nonhuman mammal according to any of (xix), (xxx) and (xxxi), of a human or animal organ-specific tissue and/or of a human or animal mammalian organ according to (xviii) for finding pharmacologically active principles and/or for identifying toxic substances.

The figures and examples below are intended to illustrate the present invention but without limiting it:

35

Fig. 1 depicts an immunoblot analysis of media supernatants of the transfection 17x4/IL15/Oligo + pcDNA3switch +/- mifepristone (example 3);

Fig. 2 depicts a diagrammatic image of the mechanism of action of the gene expression system of the invention, which can be regulated by adding an active substance.

5

The gene switch of the present invention is a chimeric protein consisting of three functional units:

10 i) a Gal4 DNA binding domain (Gal-DBD) which recognizes the gene switch binding domain UAS. The UAS sequence of the invention comprises four copies of a sequence motif of 17 nucleotides, each of which motifs can serve as a binding site for two Gal4-DBD molecules.

15 ii) a truncated ligand binding domain of the human progesterone receptor (PR-LBD) which mediates binding of the active substance mifepristone to the gene switch and causes the gene switch protein to be converted into an active conformation by way of dimerization.

20 iii) a p65 activation domain (P65-AD) which activates transcription of the target gene by means of the gene switch.

25 In the absence of mifepristone, small quantities of the gene switch are transcribed via the upstream ubiquitous weak basal minimal thymidine kinase promoter (pTK). These gene switch molecules, however, are present as monomers and thus cannot yet bind any DNA regions or initiate transcription. After addition of the active substance mifepristone, the gene switch system is activated. The ligand-bound gene switch homodimer binds to any DNA regions containing UAS sequences (such as, for example, the regulatory region upstream of the TATA promoter-regulated gene for MuTIL15-mFc) and thus activates transcription of the immuno-modulatory protein. Since, in addition, DNA regions with UAS sequences are 30 located upstream of the TK promoter of the gene switch protein gene, transcription of said gene switch protein itself is activated at the same time in an autoregulatory feedback mechanism, thus increasing the amount of the active gene switch.

35 The transplantable cells of the invention, prepared from cell lines, may furthermore contain marker genes, for example for resistances to the antibiotics neomycin (Neo-R) and hygromycin (Hygro-R). The marker genes which are regulated by an ubiquitous promoter such as, for example, the phosphoglycerate kinase promoter

(pGK) serve to select the cell for uptake of the DNA construct. The marker genes controlled by a cell type-specific promoter such as, for example, the rat insulin promoter RIP) serve to select transgenic cells of a specific cell type.

5 In order to generate the transgenic animals of the invention, the cells must contain at least the elements 1 and 2. To prepare the transgenic cells from cell lines, the cells may additionally also contain element 3.

Examples

10

To study and demonstrate the manner of action of regulated expression of the immunomodulating protein MutIL-15/mFc, two experimental models were developed:

15 a) Transgenic cell line for transplantation

In this model, stem cells were transfected with vector constructs which, in addition to the elements for regulatable expression of the MutIL-15/mFc immune modulator, contained a selection cassette which enabled a specific cell type (e.g. 20 insulin-producing cell) to be prepared (see US 5,733,727). In this way it is possible to prepare and isolate differentiated cells of a specific cell type from undifferentiated transgenic stem cells. These transgenic differentiated cells may be transplanted into a suitable recipient mouse (e.g. diabetic mouse). If the 25 transplanted animals are treated with mifepristone, the transplanted cells themselves produce MutIL-15/mFc and thus prevent their own rejection.

b) Transgenic mouse model

Transgenic mice were generated whose genome contains a construct which causes 30 regulated expression of MutIL-15/mFc via addition of mifepristone. Since MutIL-15/mFc expression is regulated by an ubiquitous promoter, the mice produced MutIL-15/mFc when stimulated by the addition of mifepristone. Organs (e.g. heart, kidney) or cells (islet cells, neuronal cells) may be removed from the transgenic animals and transplanted into another, nontransgenic mouse. When the 35 transplanted animals are treated with mifepristone, the transplanted organ/cells itself/themselves produce MutIL-15/mFc and thus prevent its/their own rejection.

Example 1: Vector cloning for a transgenic mouse model

The luciferase gene in the 17x4/pGL3 Basic vector (modified pGL3Basic vector from Promega with TATA box and 4 copies of the 17-oligomer as gene switch binding site) was replaced with the gene for a fusion protein of mutated IL-15 protein and murine Fc moiety (referred to as MutIL-15/mFc hereinbelow: Kim et al., The Journal of Immunology, 1998, 160: 5742-5748), which additionally contains a CD5 leader sequence (Jones H., Nature 323 (6086), 346-349, 1986). As a result, the CD5-MutIL-15/mFc gene is terminated by an SV40polyA sequence (vector name: 17x4/IL15). Subsequently, an oligonucleotide containing the SbfI and PmeI cleavage sites was introduced upstream of the 17-oligomers (vector name: 17x4/IL15/Oligo). Said cleavage sites served to introduce the gene for the gene switch molecule (GS = Gal4-DBD/hPR-LBD/p65-AD), including the upstream regulatory region (Gal4UAS-PTK-IVS8), which were isolated from the pswitch vector (Invitrogen, Karlsruhe, Germany) (vector name: 17x4/IL15/Oligo/GS). Alternatively and additionally, a vector was prepared into which the IVS8 intron (likewise from pswitch) was inserted between the TATA box and the Startcodon of the CD5-MutIL-15/mFc gene (vector name: 17x4/IL15/Oligo/IVS8/GS). The transitions between the segments of all cloning products were checked by sequencing.

Example 2: Vector cloning for transgenic in vitro transplants from insulin-producing cells

These vectors were prepared analogously to the vectors for the transgenic mouse model. However, these plasmids additionally also contain a fragment which comprises a neomycin resistance gene (neo) regulated by the rat insulin promoter (RIP) and a hygromycin resistance gene (hygro) regulated by the murine phosphoglycerate kinase promoter (pGK). These elements were inserted 5' below the CD5-MutIL-15/mFc gene and alternatively cloned in two transcriptional directions (vector names: 17x4/IL15/Oligo/RIPhn/GS and 17x4/IL15/Oligo/RIPnh/GS). Alternatively and additionally, vectors were prepared into which the IVS8 intron (likewise from pswitch) was inserted between the TATA box and the Startcodon of the CD5-mutIL15-mFc gene (vector names: 17x4/IL15/Oligo/RIPhn/IVS8/GS and 17x4/IL15/Oligo/RIPnh/IVS8/GS). The transitions between the segments of all cloning products were checked by sequencing.

Example 3: Checking of regulated expression and secretion of CD5-mutIL15-mFc in the vectors mentioned in examples 1 and 2

Transient transfections of cos-7 cells (DSMZ, Brunswick, Germany) and A293 cells (Quantum, Montreal, Canada) were carried out. For this purpose,  $5-7.5 \times 10^5$  cells/well were seeded on 6-well plates on the day before transfection. Transfection with 1-2  $\mu$ g DNA/well was carried out by mixing in each case 10-20  $\mu$ l of DNA at a concentration of 10 ng/ml with in each case 250  $\mu$ l of OptiMEM-I medium (Life Technologies, Karlsruhe, Germany). In a parallel experiment, in each case 2  $\mu$ l of Lipofectamine 2000 (Life Technologies, Karlsruhe # 11668-019)/ $\mu$ g of DNA were mixed with 250  $\mu$ l of OptiMEM-I medium (Life Technologies, Karlsruhe # 31985-062). Equal volumes of both mixtures were mixed and left at room temperature for 20 minutes. In the meantime, the 6-well plates containing the cells which were 50-70% confluent were washed once with PBS and then in each case 1.5 ml of growth medium/well (DMEM + 10% FCS) were added. In each case 500  $\mu$ l of the DNA/Lipofectamine mixture per well were added dropwise to said cells. Cells stimulated with mifepristone additionally received in each case 2  $\mu$ l of a  $10^{-5}$  M solution of mifepristone (Sigma, Deisenhofen, Germany; final concentration  $10^{-8}$  M) in 80% ethanol (Invitrogen, Karlsruhe, Germany). On the next day, the medium was removed and 2 ml of fresh medium (DMEM + 10% FCS) were added. The mifepristone-stimulated cells here again additionally received in each case 2  $\mu$ l of mifepristone. 3 days after transfection, the medium was removed from the cells, centrifuged to remove cell residues and subsequently frozen at -80°C for storage. The cells were washed once with PBS, scraped off into PBS, transferred to a 1.5 ml Eppendorf vessel and lysed in each case with 50  $\mu$ l of RIPA buffer (PBS containing 1% IGEPAL/Sigma, Deisenhofen I-3021, 0.5% sodium deoxycholate, 0.1% SDS, 4 mM EDTA and Complete EDTA-free Protease Inhibitor Cocktail/ Roche, Mannheim, Germany, #1873580) at 4°C for 30-60 min. The centrifuged lysates were quick-frozen in liquid nitrogen and stored at -80°C.

CD5-mutIL15-mFc secretion was analyzed with the aid of an ELISA which recognizes the murine Fc moiety of the fusion protein. To this end, 96-well plates (Nunc, Wiesbaden, Germany # 439454) were coated with in each case 100  $\mu$ l of an anti-mouse IgG2a antibody (clone R11-89, BD PharMingen, Heidelberg, Germany # 02251D-553446) at 37°C for one hour. The plates were subsequently washed three times with PBS and incubated with DMEM+10%FCS at 37°C for one hour in order to saturate unspecific binding sites. In each case 100  $\mu$ l of undiluted medium

supernatants were applied to the coated plates which were then incubated at room temperature for 1 h and subsequently washed 5× with in each case 200 µl of PBS/0.1% Tween20 and 1× with in each case 200 µl of PBS. To detect the murine Fc moiety, the enzyme-coupled HRP anti-mouse IgG 2a antibody, clone R19-15 (BD 5 PharMingen, Heidelberg # 02017E-553391), was likewise incubated with the plates again at room temperature for 1 h and the plates were then washed again as described above. The amount of bound fusion protein was visualized by a color reaction after addition of an OPD-containing substrate solution (25 ml of 0.1 M citric acid, 25 ml of 0.1 M dipotassium hydrogen phosphate, to 100 ml with H<sub>2</sub>O + 10 1 OPD tablet (Sigma Deisenhofen #P8412) + 40 µl of H<sub>2</sub>O<sub>2</sub>, 30%), and, after stopping the reaction by adding 3 M HCl, measured in an ELISA reader (µQuant, BIO-TEK Instruments Inc.) at 490 nm.

Furthermore, undiluted media supernatants were analyzed by immunoblotting. For 15 this purpose, media supernatants were mixed with Lämmli sample buffer, heated to 92°C for 5 minutes, then applied to a 12.5% strength polyacrylamide gel and fractionated electrophoretically at 150 V. The proteins were subsequently transferred to a nitrocellulose membrane (Schleicher u. Schuell, Dassel, Germany CD0564-1). The membrane was treated with 5% milk powder/PBS/0.1% Tween20 20 to saturate unspecific binding sites. It was then incubated with a 1:500 dilution of mouse anti-human IL15 antibody (Becton-Dickinson, Heidelberg #554712) at 4°C for 16 hours, extensively washed with PBS/0.1% Tween20 and then treated with a 1:3000 dilution of a peroxidase-coupled sheep anti-mouse antibody (Amersham, Freiburg, Germany #NA9310) at room temperature for one hour. After extensive 25 washing, the membrane was treated with detection reagent (NEN; Bad Homburg, Germany #NE103E) at room temperature for 5 minutes and the color reaction was detected by applying an X-ray film.

The analyses of media supernatants of transfected cells with and without addition 30 of mifepristone revealed the following result:

The following transfections were carried out:

1. mCD5.6 (positive control for MutIL-15/mFc secretion, vector containing 35 CD5-MutIL-15/mFc under the control of a CMV promoter)
2. 17×4/IL15/Oligo +/- mifepristone (negative control without gene switch)

3. 17x4/IL15/Oligo + pcDNA3switch +/- mifepristone
4. 17x4/IL15/Oligo/GS +/- mifepristone
5. 17x4/IL15/Oligo/GS + pcDNA3switch +/- mifepristone
6. 17x4/IL15/Oligo/IVS8/GS +/- mifepristone
- 10 7. 17x4/IL15/Oligo/IVS8/GS + pcDNA3switch +/- mifepristone

No significant expression of MutIL-15/mFc was observed in cells which had been transfected with the control vector without gene switch molecule (2.) and in cells which had not been stimulated with mifepristone. After transfection with constructs which contained merely the auto-regulated gene switch molecule (4. + 6.), only a slight increase in MutIL-15/mFc secretion after mifepristone stimulation was detected with the aid of the ELISA. This was presumably due to insufficient formation of active gene switch molecules by auto-regulatory gene activation in the course of the transient transfection. Therefore, the amount of gene switch was increased externally by way of cotransfection with the pcDNA3switch vector (gene switch under the control of the CMV promoter) which effects consistently high gene switch production. In these cotransfections, the media supernatant of cells which had been transfected both with gene switch-free (3.) and with gene switch-containing (5. + 7.) constructs contained, after mifepristone treatment, an increased amount of MutIL-15/mFc which was detected by ELISA.

Table 1: Measurement of MutIL-15/mFc in media supernatants of transiently transfected A293 cells (average of duplicates)

	Construct	pcDNA3switch	mifepristone	M 490 Corr.
1	mCD5.6	-	-	1.750
2	17x4/IL15/Oligo	-	+	0.006
3	17x4/IL15/Oligo	+	-	<0.000
	17x4/IL15/Oligo	+	+	0.067
4	17x4/IL15/Oligo/GS	-	-	<0.000
	17x4/IL15/Oligo/GS	-	+	0.006
5	17x4/IL15/Oligo/GS	+	-	0.004
	17x4/IL15/Oligo/GS	+	+	0.019
6	17x4/IL15/Oligo/IVS8/GS	-	-	0.006
	17x4/IL15/Oligo/IVS8/GS	-	+	0.009
7	17x4/IL15/Oligo/IVS8/GS	+	-	0.010
	17x4/IL15/Oligo/IVS8/GS	+	+	0.022

5 Immunoblot analyses of media supernatants of transfection 3. (fig. 1) likewise confirmed production of an increased amount of MutIL-15/mFc (50 kDa band) after mifepristone stimulation (lane 1), in comparison with unstimulated cells (lane 2).

10 These results prove that transcription and secretion of MutIL-15/mFc fusion protein can be induced by addition of mifepristone in cells transfected with the vectors introduced above (e.g. 17x4/IL15/Oligo/IVS8/GS). This proved the functionality of regulated MutIL-15/mFc expression.

15 Example 4: Checking of regulated expression and functionality of the gene switch protein (Gal4UAS-PTK-IVS8-Gal4-DBD/hPR-LBD/p65-AD) in the vectors mentioned in examples 1 and 2

20 As mentioned before, the number of active gene switch molecules that in transiently transfected cells producing only auto-regulated gene switch (transfection with 17x4/IL15/Oligo/GS and 17x4/IL15/Oligo/IVS8/GS) was low.

Since the ELISA is not sensitive enough in order to detect expression of MutIL-15/mFc at the single cell level, cotransfections with the pGene/V5-His/lacZ plasmid (Invitrogen, Karlsruhe) were carried out. This vector contains a lacZ gene whose transcription is likewise regulated by gene switch binding sites, meaning that  $\beta$ -galactosidase is produced only in those cells which simultaneously contain active gene switch due to mifepristone stimulation. Said gene switch was provided by a second vector (in this case the constructs with auto-regulated gene switch).  $\beta$ -Galactosidase was detected by way of a substrate reaction as a blue precipitate in individual cells. It was thereby possible to detect a function of the vector constructs at a substantially higher sensitivity.

As described in example 3, A293 cells were transfected with the following constructs and then partly stimulated with mifepristone:

- 15 1. pCMV $\beta$  (positive control for lacZ, vector containing the lacZ gene under the control of a CMV promoter)
2. 17 $\times$ 4/IL15/Oligo + pGene/V5-His/lacZ +/- mifepristone (negative control without gene switch)
- 20 3. 17 $\times$ 4/IL15/Oligo/GS + pGene/V5-His/lacZ +/- mifepristone
4. 17 $\times$ 4/IL15/Oligo/IVS8/GS + pGene/V5-His/lacZ +/- mifepristone
- 25 5. pcDNA3switch + pGene/V5-His/lacZ +/- mifepristone (positive control with constitutively high gene switch)
6. pGene/V5-His/lacZ-mifepristone (negative control without gene switch)

30 On the third day after transfection, the cells were fixed with ice-cold methanol at -20°C for 10 min, washed 3 $\times$  with PBS and then treated with lacZ staining solution (60  $\mu$ l of 400 mM potassium ferricyanide, 60  $\mu$ l of 400 mM potassium ferrocyanide, 60  $\mu$ l of 200 mM MgCl<sub>2</sub>, 300  $\mu$ l of 20 mg/ml X-Gal, 5.52 ml of PBS) at 37°C for 2.5 h.

35 Evaluation by means of light microscopy of the transfected cells gave the following result (table 2):

Table 2: Intensity of  $\beta$ -galactosidase staining of transiently transfected A293 cells

	Construct	Cotransfection with pGene/V5-His/lacZ	Addition of mifepristone	Result
1	pCMV $\beta$	-	-	***
2	17 $\times$ 4/IL15/Oligo	+	-	(*)
	17 $\times$ 4/IL15/Oligo	+	+	*
3	17 $\times$ 4/IL15/Oligo/GS	+	-	(*)
	17 $\times$ 4/IL15/Oligo/GS	+	+	***
4	17 $\times$ 4/IL15/Oligo/IVS8/GS	+	-	-
	17 $\times$ 4/IL15/Oligo/IVS8/GS	+	+	***
5	pcDNA3switch	+	-	***
	pcDNA3switch	+	+	*****
6	pGene/V5-His/lacZ	-	-	-

\*: degree of staining intensity

5

No significant blue staining of cells was observed without gene switch (2.). In cells which had been transfected with auto-regulated gene switch (3. + 4.), stimulation with mifepristone caused distinct blue staining of 3-5% of the cells.

10 Since this low number of cells produces only a very small amount of MuIL-15/mFc, said amount is below the detection limit of the ELISA. It is, however, possible for lacZ staining to record and detect the gene switch activity also in individual cells.

15 These experiments prove the ability of the activated gene switch which is provided by the 17 $\times$ 4/IL15/Oligo/GS and 17 $\times$ 4/IL15/Oligo/IVS8/GS constructs after stimulation with mifepristone to effect transcription of lacZ. These data thus prove the functionality of the auto-regulated gene switch of the abovementioned constructs.

20

Example 5: Preparation and characterization of transgenic mice

50  $\mu$ g of DNA of the constructs 17 $\times$ 4/IL15/Oligo/GS and 17 $\times$ 4/IL15/Oligo/IVS8/GS were cut with the restriction enzymes Eco47III and

NotI and the desired 4800 bp and, respectively, 4924 bp fragments were purified. Said fragments were then injected into the pronuclei of C3HeB/FeJ mice and the embryos were subsequently implanted into pseudopregnant females. Genomic DNA was extracted from the mice obtained and examined for integration of the transgene with the aid of PCR analysis. The following primers were used in said PCR analysis: GS-IL15FW.2 (5'-TAT GGC TTC TGA GGC GGA AAG AAC CAG C - 3') and GS-L15 RV.3 (5'-G CAG AGA CCC CAT GGG CAT GGT GGC TAG - 3'). Accordingly, the PCR products obtained are 211 bp (without intron) and 335 bp (with IVS8 intron), respectively, in length.

10

Of 44 mice obtained from oocyte injection of the 17 $\times$ 4/IL15/Oligo/GS construct, 16 animals (36%) were transgenic.

15

The founder animals (F0 generation) were mated with wild type DBA/2 mice and the F1 progeny obtained were again examined for genomic presence of the transgene. The transgenic F1 animals were subsequently examined for regulated expression of MutIL-15/mFc. At the age of approx. 8-16 weeks, the animals were injected intraperitoneally with 250  $\mu$ g/kg mifepristone dissolved in sesame oil (Sigma, Deisenhofen M 8046) every other day, three times in total. The animals were sacrificed one day after the last injection and RNA and protein were extracted from the tissues. Gene switch protein and immune modulator expression in the tissues was then analyzed with the aid of ELISA and Western blot. The amount of RNA is determined by means of a quantitative reverse transcription PCR (RT-PCR) in order to determine the amount of transcribed gene switch and immune modulator.

20

In each case, 1  $\mu$ g of RNA is transcribed with the aid of Expand Reverse Transcriptase (Roche, Mannheim) into cDNA according to the manufacturer's instructions. This is followed by studying quantitatively expression of the gene switch and of the immune modulator MutIL15/mFc with the aid of the Light Cycler Fast Start DNA Master SYBR Green Kit (Roche, Mannheim). The PCR conditions for detecting the immune modulator are as follows:

denaturation: 95°C, 600 sec

cycles: 95°C 15 sec, 60°C 5 sec, 72°C 10 sec.

Primer CD5.6-FW: 5'-CCTGCTGGGGATGCTGGTC

30

Primer CD5.6-RV: 5'-TTTCCTCCAGTTCCACATTC

MgCl<sub>2</sub>: 3 mM

The PCR conditions for detecting the gene switch are as follows:

denaturation: 95°C, 600 sec

cycles: 95°C 15 sec, 53°C 5 sec, 72°C 10 sec.

Primer GS-FW: 5'-GACTTAAAAAGCTCAAGTGCTCCAAAG

Primer GS-RV: 5'-TATATCCTGTAAAGAATCCAT

5 MgCl<sub>2</sub>: 3 mM

Moreover, blood is taken from the animals at regular intervals before and during mifepristone treatment, and the amount of MutIL-15/mFc contained therein is determined via ELISA.

10

Mice of the same age and of the same transgenic line which had not been treated with mifepristone previously are used for comparison.

15

Alternatively, the transgenic mice are prepared in a two-step process. First, two different lines of transgenic mice are generated which express either only the gene switch molecule (lines "A") or only the immune modulator regulated by the gene switch binding site (lines "B"). Of the transgenic mice strains of the A lines obtained, those animals are selected which express suitable amounts of gene switch molecule and mated, in a second step, with transgenic animals of the B lines. The 20 progeny obtained are then examined for simultaneous expression of the two transgenes. In this way, double-transgenic lines "A/B" are obtained which express immune modulator regulated by gene switch molecules.

25

50 µg of DNA of the constructs pswitch (for lines "A") or 17×4/IL15/Oligo and 17×4/IL15/Oligo/IVS8 (for lines "B") are cut with suitable restriction enzymes and the desired fragments are purified. Said fragments are then injected into the pronuclei of C3HeB/FeJ mice and the embryos are subsequently implanted into pseudopregnant females. Genomic DNA is extracted from the mice obtained and examined for integration of the transgene with the aid of PCR analysis.

30

#### Example 6: Transplantation of islets from donor organs of transgenic mice

Islet transplantation involves injecting islets isolated from pancreases of mice under the renal capsule of diabetic mice (Ferrari-Lacraz et al., The Journal of Immunology, 2001, Vol. 167 pp. 3478-3485). Donor islets are isolated from transgenic mice of the DBA/2J strain which express MutIL-15/mFc under the control of the gene switch. The donor mice are pretreated with mifepristone (250 µg/kg) so as to detectably produce MutIL-15/mFc on the day of organ removal. To prepare the islets, donor pancreases are perfused *in situ* with type IV

collagenase (2 mg/ml; Worthington Biochemical Corp.). After digestion at 37°C for 30 minutes, the islets are purified via discontinuous Ficoll gradient and subsequently cultured in RPMI1640 medium (Gibco, Karlsruhe) containing 5.6 mM glucose. 300-400 islets are then transplanted under the renal capsule of the recipient.

The recipient is a 6-10 week old nontransgenic B6AF1 mouse in which diabetes has been induced by injecting intraperitoneally the beta-cell toxin Streptozotocin (225 mg/kg; Sigma, Deisenhofen). Syngeneic islets (i.e. islets of B6AF1 mice) are transplanted as control into the diabetic mice.

Pancreatic islets are transplanted aseptically under the left renal capsule. For this purpose, the mouse is anesthetized and a cut is performed under the left costal arch. The left kidney is mobilized out of the abdominal cavity and a short incision is made in the lower pole of the renal capsule. Using a blunt sterile canula, a pocket is formed under the renal capsule. The islets are then injected into this cavity by means of a sterile pipette tip. Subsequently, the kidney is put back into the abdominal cavity and the abdomen is closed. From the day of transplantation onward, the recipient animals are treated every other day with mifepristone (250 µg/kg) so that they continuously produce MutIL-15/mFc.

Allotransplant function is monitored via regular blood glucose measurements (Accu-Check III; Boehringer Mannheim, Mannheim, Germany). Before and after induction of diabetes and after islet transplantation, blood is removed from the tail vein or the retrobulbar venous plexus of the animals at regular intervals and the blood sugar level is determined. Additional controls are carried out between the takings of blood, using urine test strips. The primary transplant function is defined as a blood sugar content below 11.1 mmol/l (200 mg/dl) on day 3-5 post transplantation. The transplant is regarded as rejected, if the blood sugar level has increased to more than 500 mg/dl on at least two successive days, after a primary transplant function is observed.

As a control, islet transplants are carried out in which the animals either do not receive any mifepristone and thus also do not express any gene switch-regulated MutIL-15/mFc or in which the animals that are treated with external addition of MutIL-15/mFc.

The animals treated in the manner described can, after transplantation of islet cells obtained from transgenic donor mice, better regulate their blood sugar level and exhibit reduced rejection of the cell transplants after treatment with mifepristone.

Example 7: Heterotopic heart transplantation

Heterotopic heart transplantation involves connecting the donor heart with the large vessels in the abdomen of the recipient (Ono et al., J. Cardiovasc. Surg. 1969, 5 Corry et al., Transplantation 1973). Donor hearts are isolated from transgenic mice of the DBA/2J strain which express MutIL-15/mFc under the control of the gene switch. The donor mice are pretreated with mifepristone (250 µg/kg) so as to detectably produce MutIL-15/mFc on the day of organ removal. The vena cava of the anesthetized mice is isolated and heparin (400 U/kg) is injected to be 10 distributed over the entire circulation. The animals are subsequently bled by cleaving the abdominal vessels and the heart is exposed with the aid of a median sternotomy. The aorta and the pulmonary vessels are isolated and cleaved and the pulmonary veins and the vena cava are ligated en masse with 4-10 Tevdek (Deknatal, Queens Village). The heart is stored in physiological saline 15 (Physiolsol, Abbot Laboratories, Illinois, USA) at 4°C immediately after its removal. The inferior vena cava and abdominal aorta of the recipient (6-8 week old nontransgenic B6AF1 mouse) are exposed and the vessels are provided with loosely fitted vascular clamps. The end of the donor aorta is connected with the side of the recipient abdominal aorta, by an 8-0 Prolene suture. The pulmonary 20 artery of the donor is fused to the vena cava of the recipient in the same manner. The vascular clamps are removed and the donor heart is heated with Ringer's lactate solution at 37°C so that the heart starts contracting again spontaneously. From the day of transplantation onward, the recipient animals are treated every 25 other day with mifepristone (250 µg/kg) so that they continuously produce MutIL-15/mFc. Transplant survival is checked every other day by palpating the beating heart through the abdominal wall and evaluated on a scale from 1+ to 4+ on the basis of the strength and rate of the pulse. The heart is regarded as rejected when heart muscle contractions are no longer palpable. Rejection is prevented if the donor hearts beat over a longer period than hearts in untreated control animals. As 30 a control, heart transplantations are carried out in which the animals either do not receive any mifepristone and thus also do not express any gene switch-regulated MutIL-15/mFc or in which the animals that are treated with external addition of MutIL-15/mFc.

This example shows that the heterotopic heart transplants remain functional for a 35 longer time in those animals treated with mifepristone, in comparison with the untreated animals.

Example 8: Preparation of transgenic ES cell lines

100 µg of DNA of the constructs 17x4/IL15/Oligo/RIPnh/GS, 17x4/IL15/Oligo/RIPhn/GS, 17x4/IL15/Oligo/RIPhn/IVS8/GS and 17x4/IL15/Oligo/RIPnh/IVS8/GS were cut with the restriction enzymes Eco47III and NotI, the fragments were purified and resuspended in at least 100 µl of sterile PBS. The constructs 17x4/IL15/Oligo/RIPhn/GS and 17x4/IL15/Oligo/RIPhn/IVS8/GS were then introduced into murine ES cells of the SVJ129 or R1 line by means of electroporation. For this purpose, exponentially growing murine ES cells were trypsinized, separated and counted. Approx.  $3 \times 10^7$  cells were washed twice with 5-10 ml of cold PBS and the cell pellet was then taken up in cold PBS so that the final volume, including DNA, is 800 µl. The digested DNA was subsequently added to the cells, the solution was mixed and incubated on ice for at least 10 minutes. The cell/DNA mixture was filled into precooled electroporation cuvettes with a 0.4 cm electrode slit (BioRad, Munich, Germany) on a sterile workbench. The electroporation was carried out using a Genepulser 2 apparatus (BioRad, Munich) at 0.8 kV and 3 µF. The cells were then mixed with ES cell medium (DMEM containing 20 mM Hepes, 15% heat-inactivated FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.1 mM non essential amino acids, 0.1 mM mercaptoethanol,  $10^3$  U/ml leukemia inhibitory factor) and distributed evenly on ten 10 cm cell culture dishes coated with 0.1% gelatin. On the next day, selection was started by adding 200 µg/ml hygromycin (Sigma, Deisenhofen H 3274) in ES cell medium. The cells were selected for uptake of the construct for approx. 5-9 days and individual transgenic clones were isolated manually on the sterile workbench and transferred to 96-well plates. The cells were passaged before reaching confluence and successively replated on 48-well plates, 24-well plates, 6-well plates and 10 cm dishes.

After addition of 10 nM mifepristone, the cell clones were examined for regulatable expression of the gene switch and MutIL-15/mFc by means of quantitative RT-PCR or of MutIL-15/mFc with the aid of ELISA and Western blot assays (as described above in examples 3 and 4).

Alternatively, the transgenic ES cells are prepared in a 2-step process. First, transgenic ES cells are generated which express only the gene switch molecule in a regulated manner. Of the transgenic cell lines obtained, those clones are selected which express suitable amounts of gene switch molecule. In a second step, these clones are supertransfected with DNA constructs encoding the immune modulator regulated by a gene switch binding site. The double-transgenic lines obtained are, as described in example 5, examined by means of RT-PCR for simultaneous

expression of the two transgenes. In this manner, double-transgenic ES cells are obtained which express the gene switch molecule-regulated immune modulator.

100 µg of DNA of the gene switch construct are cut with suitable restriction enzymes, the desired fragment is purified, resuspended in sterile PBS and 5 introduced into ES cells by electroporation, as described above. Since the construct imparts a resistance to the antibiotic zeocin, the cells may be selected for successful transfection by treatment with said antibiotic. Transgenic clones are isolated from the cells obtained and expression of the gene switch molecule as a function of mifepristone addition is studied by quantitative RT-PCR. Those clones 10 which express suitable amounts of the gene switch molecule are, in a second step, supertransfected again by electroporation with purified Eco47III/NotI fragments of the constructs 17×4/IL15/Oligo/RIPhn and 17×4/IL15/Oligo/RIPhn/IVS8 and selected for uptake of the second transgene by means of hygromycin treatment. 15 The simultaneous integration of both transgenes is checked by means of PCR analysis of the genomic DNA.

The double-transgenic cell clones obtained are, after addition of 10 nM mifepristone, examined for regulatable expression of the gene switch and MutIL-15/mFc by means of quantitative RT-PCR or of MutIL-15/mFc protein with the aid of ELISA and Western blot assays, as described already above in example 5. 20 Subsequently, those clones are selected from the cell lines obtained, which produce and secrete sufficient quantities of the immune modulator MutIL-15/mFc only after addition of mifepristone.

Example 9: Transplantation of insulin-producing cells prepared from transgenic ES 25 cells

Prior to transplantation, insulin-producing cells which express MutIL-15/mFc under the control of the gene switch are prepared from undifferentiated hygromycin-resistant ES cell clones, Soria et al. (Diabetes. 2000 Feb.; 49(2): 157-62.

30 The insulin-producing cells are treated with mifepristone before transplantation so as to produce sufficient amounts of MutIL-15/mFc.

Subsequently, 1 million insulin-producing cells are injected into C57BL/6 mice which are diabetic due to streptozotocin treatment, either under the renal capsule (as described in example 7) or into the spleen (Soria et al. (Diabetes. 2000 Feb.; 35 49(2): 157-62). From the day of transplantation onward, the recipient animals are treated every other day with mifepristone (250 µg/kg) so that they continuously produce MutIL-15/mFc.

The allotransplant function is monitored via regular blood glucose measurements (Accu-Check III; Boehringer Mannheim, Mannheim). The primary transplant function is defined as a blood sugar content of below 11.1 mmol/l (200 mg/dl) on 5 day 3-5 post transplantation. The transplant is regarded as rejected if the blood sugar level increases to more than 500 mg/dl on at least two successive days, if a primary transplant function is observed previously.

As a control, insulin-producing cells which have been prepared from the same cell 10 clone but have not been pretreated with mifepristone and thus also do not express any gene switch-regulated MutIL-15/mFc are transplanted into recipient animals. Alternatively, it is also possible to use insulin-producing cells which contain the MutIL-15/mFc construct but not the gene switch.

15 Animals which are treated with external addition of MutIL-15/mFc after having received insulin-producing cells derived from stem cells serve as a further control.

Studies of the blood sugar level of animals which have received insulin-producing 20 cells prepared from stem cells indicate that cell transplants are functionally active for a relatively long time in animals treated with mifepristone.